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Original Research

Role of Janus-associated kinases in somatostatin analog preconditioning of human umbilical-vein endothelial cells

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Abstract

Background: Somatostatin (SST) has been proven to have cardioprotective effects, but its effects on endothelial cells has not yet well studied. **Aim:** To confirm the phenomenon of SST-induced preconditioning (PC) and the cellular mechanisms involved, we designed an *in vitro* study to investigate the effects of SST analogs on tumor necrosis factor (TNF)- α -induced endothelial nuclear factor (NF)- κ B activation with subsequent interleukin (IL)-6 and IL-8 release.

Methods: Experiments were performed on primary human umbilical-vein endothelial cells (HUVECs). IL-6 and IL-8 were measured using commercial enzyme-linked immunosorbent assay kits. An electrophoretic mobility shift assay (EMSA) was used to demonstrate NF- κ B activation. The effects of pretreatment with octreotide, an SST analog, and/or *N*-acetyl-cysteine (NAC) were tested.

Results: TNF- α stimulated IL-6 and IL-8 production from HUVECs. SST PC using octreotide at concentrations $>10^{-8}$ M attenuated TNF- α -induced IL-6 and IL-8 release, but NAC did not inhibit SST-treated endothelial cells stimulated by TNF- α . EMSA revealed that TNF- α treatment was associated with activation of NF- κ B, which could be inhibited by SST PC. By contrast, wortmannin and AG-490 reversed the inhibitory effects of octreotide on TNF- α -induced NF- κ B activation, but neither had any definite effects on TNF- α -induced NF- κ B activation in the absence of octreotide. Western blots confirmed that octreotide modulated I κ k at 10^{-8} M.

Conclusion: SST PC modulates I κ k in a PI3K- and JAK-2-dependent pathway, which in turn attenuates activation of NF- κ B induced by TNF- α in HUVECs.

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1. Introduction

We previously demonstrated many modes of preconditioning (PC) in experiments to show cardioprotective effects.^{1–3} PC represents a powerful intervention that reduces infarct size after ischemia–reperfusion or other pretreatments such as stimulation or induction of the adrenergic system,⁴ adenosine receptors,⁵ ATP-sensitive potassium channels,⁶ heat shock proteins,⁷ and oxidative stress.⁸ Some investigators further studied the

possible effector cells of such PC. Although we proposed that neutrophils are target cells for PC,¹ endothelial cells are still the main effectors cells used in most research.^{2,9–11}

Endothelial cells are associated with many pathophysiological changes that may be related to PC. Theoretically, endothelial PC should be closely related to the mechanisms of PC mentioned above.⁶ However, the real link between endothelial PC and myocardial PC deserves further investigation. One of the mechanisms involved in endothelial PC is oxidative stress. It has been suggested that oxidative stress and cellular redox status are significant modulators of intracellular signaling.¹² Zahler et al⁹ demonstrated that endothelial cells can be preconditioned by transient intracellular redox stress to reduce

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responsiveness to an inflammatory mediator. Translocation of the transcription factor nuclear factor (NF)- κ B may facilitate novel approaches directed at alleviating reperfusion damage. Because it has been demonstrated that mechanical stretch can activate matrix metalloprotease expression via tumor necrosis factor (TNF)- α and the NF- κ B pathway,¹³ NF- κ B may be the central mediator of endothelial PC, whereas mitochondrial ATP-sensitive potassium (mitoK_{ATP}) channels are believed to be the core mediators of myocardial PC. We previously demonstrated a reduction in infarct size due to SST PC,³ so it seemed reasonable to investigate if SST PC could also decrease damage due to TNF- α as an additional protective mechanism.

We thus designed an *in vitro* study to investigate the phenomenon of SST-induced PC and the signaling mechanisms involved in human endothelial cells.

2. Methods

2.1. Primary culture of human umbilical-vein endothelial cells

Endothelial cells were cultured from human umbilical veins (<24 hours post partum) using a modification of the method described by Jaffe et al.¹⁴ After cannulation at both ends, cords were flushed with 120 mL of HEPES-buffered saline (10 mM) and incubated with 0.2% collagenase (Sigma, St. Louis, MO, USA) at 37°C for 15 minutes. HUVECs were grown to 100% confluence on gelatin-coated T25 flasks (Becton Dickinson, Franklin Lakes, NJ, USA) in medium 199 supplemented with 20% fetal calf serum, 100 IU/mL penicillin, 100 µg/mL streptomycin, 10 mM HEPES, and 0.2 M L-glutamine (Sigma) at pH 7.4 and 37°C. Purification was determined by flow cytometry (FACS, Becton Dickinson) with a factor VIII-related antigen antibody. Endothelial cells in the first passage were used for experiments.

2.2. SST PC experimental protocol

In PC experiments, medium was removed from confluent HUVECs and replaced with PBS containing octreotide (an SST analog at 10⁻¹⁰ M, 10⁻⁸ M, or 10⁻⁶ M) for 5 minutes at 37°C. The supernatant was then removed and cells were covered with standard medium again and incubated with TNF- α (2.5 ng/mL) for 4 hours. The supernatant was then sampled for measurement of IL-6 and IL-8. Cells were detached. Time-matched controls were treated with neither octreotide nor TNF- α . Further experimental groups consisted of cells treated with octreotide (5 minutes) or TNF- α (4 hours) alone. For comparison, experiments were repeated in cells pretreated with the intracellular radical scavenger *N*-acetyl-cysteine (NAC, 1 mM) for 30 minutes in comparative groups. After incubation, the cells were washed to remove all extracellular NAC and then subjected to the aforementioned procedures. Some experiments were terminated at 5 minutes or 30 minutes after SST treatment for analysis of subcellular NF- κ B localization.

2.3. Interleukin ELISA

Levels of the cytokines IL-6 and IL-8 were determined in cell culture supernatants using commercial enzyme-linked immunosorbent assay (ELISA) kits (Endogen, Woburn, MA, USA). To determine IL-8, samples were diluted 1:100. Sample aliquots of 50 µL per well were used in all cases and measurements were performed according to the manufacturer's instructions. In brief, ELISA plates were incubated with standards or samples for 60 minutes. Unbound cytokine was removed by washing three times, and a second antibody (labeled enzymatically for photometric detection) was added. After further incubation and removal of unbound antibody by washing, the plates were analyzed photometrically using a microplate reader (Dynatech, Guernsey, UK) at 450 nm. As an indicator of cell death, release of lactate dehydrogenase into the supernatant was also measured photometrically at 490 nm.

2.4. NF- κ B electrophoretic mobility shift assay

The protein content of nuclear extracts was determined using a BioRad (Hercules, CA, USA) protein assay. Oligonucleotide sequences were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A double-strand NF- κ B consensus oligonucleotide probe (5'-AGT TGA GGG GAC TTT CCC AGG-3') was labeled with ³²P and 4 µg of nuclear protein in 2 µL of nuclear extract was mixed with 20 pmol of [γ -³²P] dATP-labeled consensus or mutant oligonucleotide in a total volume of 20 µL at room temperature for 30 minutes. The samples were then resolved on a 4% polyacrylamide gel. Gels were dried and imaged by autoradiography at -70°C.

2.5. Signaling pathways for TNF- α and SST PC

To elucidate the underlying signaling mechanisms, HUVECs were subjected to different pretreatment protocols, including AG-490 [100 µM; a Janus-associated kinase (JAK)-2 antagonist], PD98059 [50 µM; a p42/p44 mitogen-activated protein kinase (MAPK) inhibitor], genistein (40 µM; a tyrosine kinase inhibitor) and wortmannin (50 nM; a phosphoinositide-3-kinase (PI3K) inhibitor).

2.6. I κ B protein expression

HUVECs were lysed in a reducing Triton lysis buffer (Tris 1 mM, NaCl 50 mM, Triton X, sodium vanadate 5 mM, NaF 50 mM, Na pyrophosphate 30 mM, DTT 1 mM, PMSF 1 mM, leupeptin 10 µM, pepstatin 10 µM). Nuclear and cytosolic fractions were separated by centrifugation (10,000 g for 5 seconds) and the membrane fraction was removed by centrifugation at 10,000 g (4°C) for 15 minutes. The protein concentration in nuclear and cytosolic lysates was determined using a detergent-compatible assay (BCA, Pierce, Rockford, IL, USA). Aliquots (40 µg) of protein were separated on a 12% polyacrylamide gel, transferred to a nitrocellulose membrane (Hybond-enhanced chemiluminescence, Amersham, Braunschweig, Germany), blocked with buffer

containing 5% nonfat dry milk, and incubated with 2 $\mu\text{g/mL}$ primary antibody (anti-I κ B; Santa Cruz Biotechnology). After washing four times with buffer containing 1% nonfat dry milk, the membranes were exposed to 0.2 $\mu\text{g/mL}$ secondary antibody labeled with horseradish peroxidase (Santa Cruz Biotechnology). The membranes were then incubated with an enhanced chemiluminescence kit (Pierce) for 1 minute and exposed to X-ray film. The films were analyzed using a GelDoc 1000 video system (BioRad) and the optical density of the bands was calculated using Molecular Analyst software (BioRad). Owing to considerable interindividual variability, values are expressed as percentage changes compared to the control (100%). Analogous experiments were performed on the cytoplasmic fraction as a control for the purity of the nuclear and cytoplasmic preparations.

2.7. Data analysis

All values are expressed as mean \pm SEM. Densitometry data for Western blotting and EMSA were analyzed using a two-tailed paired *t* test or two-way analysis of variance followed by the Bonferroni test when appropriate (SPSS 10.0 for Windows, SPSS, Chicago, IL, USA). A *p* value <0.05 was considered to be statistically significant.

3. Results

3.1. SST PC effects on TNF- α -induced endothelial IL-6 and IL-8 release

As shown in Fig. 1, TNF- α stimulated significant secretion of IL-6 (8.5 ± 1.9 pg/mL vs. 10.1 ± 0.5 pg/mL, $p < 0.05$) and IL-8 (1080 ± 200 pg/mL vs. 1420 ± 50 pg/mL, $p < 0.05$) by HUVECs. NAC decreased detectable IL-6 (6.6 ± 0.7 pg/mL vs. 10.1 ± 0.5 pg/mL, $p < 0.01$) and IL-8 (1100 ± 200 pg/mL

vs. 1420 ± 50 pg/mL, $p < 0.05$). SST PC had significant inhibitory effects on TNF- α -induced IL-6 and IL-8 secretion at octreotide concentrations $>10^{-8}$ M (IL-6: 6.5 ± 0.6 pg/mL vs. 10.1 ± 0.5 pg/mL, $p < 0.01$; IL-8: 1120 ± 210 pg/mL vs. 1420 ± 50 pg/mL, $p < 0.05$). However, NAC had no synergistic effects with SST (e.g., at 10^{-8} M octreotide, IL-6: 6.5 ± 0.6 pg/mL without NAC vs. 7.0 ± 0.7 pg/mL with NAC, $p > 0.05$; IL-8: 1120 ± 210 pg/mL with NAC vs. 1180 ± 230 pg/mL without NAC, $p > 0.05$).

In summary, ELISA revealed that TNF- α induced endothelial release of IL-6 and IL-8, which was inhibited by pretreatment with NAC. SST PC attenuated TNF- α -induced IL-6 and IL-8 release, but did not inhibit NAC-treated endothelial cells stimulated by TNF- α (Fig. 1).

3.2. SST PC inhibits TNF- α -induced NF- κ B activation via a PI3K- and JAK-2-dependent pathway

EMSA revealed that TNF- α induced NF- κ B activation compared to control cells (Lanes 2 and 3, Fig. 2). Neither AG-490 (a JAK-2 antagonist), PD98059 (a MAPK inhibitor), genistein (a tyrosine kinase inhibitor) nor wortmannin (a PI3K inhibitor) had any effects on TNF- α -induced NF- κ B activation (Lanes 10–13, Fig. 2).

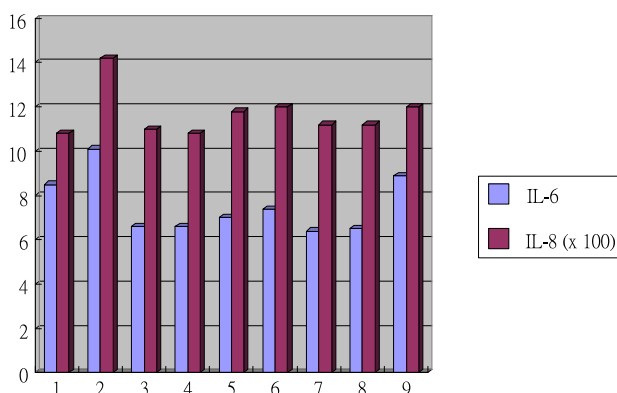


Fig. 1. Measurement of IL-6 and IL-8 from human umbilical-vein endothelial cells. Lane 1: control; lane 2: TNF- α alone; lane 3: TNF- α and pretreatment with *N*-acetyl-cysteine (NAC); lane 4: TNF- α , pretreatment with NAC and octreotide (10^{-6} M); lane 5: TNF- α , pretreatment with NAC and octreotide (10^{-8} M); lane 6: TNF- α , pretreatment with NAC and octreotide (10^{-10} M); lane 7: TNF- α and pretreatment with octreotide (10^{-6} M); lane 8: TNF- α and pretreatment with octreotide (10^{-8} M); lane 9: TNF- α and pretreatment with octreotide (10^{-10} M).

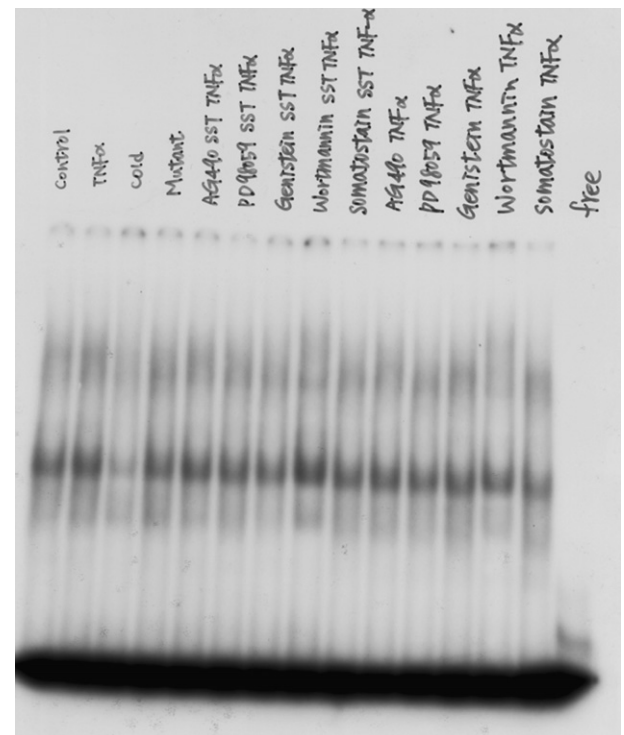


Fig. 2. Electrophoretic shift mobility assay showing different degrees of NF- κ B activation. Lane 1: control; lane 2: TNF- α alone; lane 3: cold; lane 4: TNF- α and pretreatment with AG-490 (100 μM); lane 5: TNF- α and pretreatment with PD98059 (50 μM); lane 6: TNF- α and pretreatment with wortmannin (50 nM); lane 7 (A + S): TNF- α , pretreatment with AG-490 (100 μM) and octreotide (10^{-8} M); lane 8 (P + S): TNF- α , pretreatment with PD98059 (50 μM) and octreotide (10^{-8} M); lane 9 (W + S): TNF- α , pretreatment with wortmannin (50 nM) and octreotide (10^{-8} M); lane 10 (S1): TNF- α and pretreatment with octreotide (10^{-8} M); lane 11 (S2): TNF- α and pretreatment with octreotide (10^{-10} M).

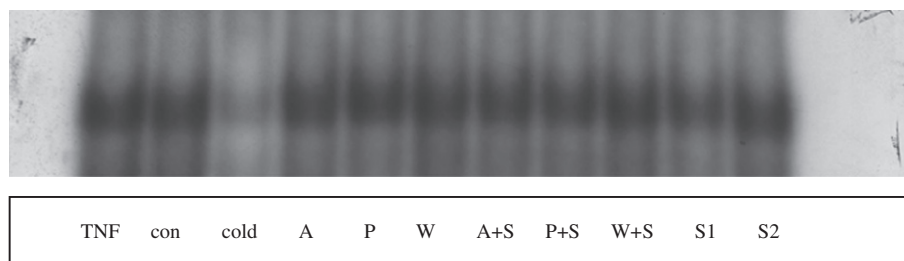


Fig. 3. Western blots for I κ c levels in HUVECs in the absence or presence of octreotide (10^{-8} M), AG-490 (100 μ M), and wortmannin (50 nM). Lane 1: TNF- α alone; lane 2: control; lane 3: cold; lane 4: mutant; lane 5: TNF- α , pretreatment with AG-490 (100 μ M) and octreotide (10^{-8} M); lane 6: TNF- α , pretreatment with PD98059 (50 μ M) and octreotide (10^{-8} M); lane 7: TNF- α , pretreatment with genistein (40 μ M) and octreotide (10^{-8} M); lane 8: TNF- α , pretreatment with wortmannin (50 nM) and octreotide (10^{-8} M); lane 9: TNF- α , pretreatment with octreotide (10^{-8} M); lane 10: TNF- α and pretreatment with AG-490 (100 μ M); lane 11: TNF- α and pretreatment with PD98059 (50 μ M); lane 12: TNF- α and pretreatment with genistein (40 μ M).

However, SST PC attenuated TNF- α -induced NF- κ B activation (Lanes 2 and 9, Fig. 3). In addition, wortmannin markedly reversed the inhibitory effects of SST PC on TNF- α -induced NF- κ B activation (Lanes 8 and 9, Fig. 2). AG-490 also reversed the effects (Lanes 5 and 9, Fig. 2). This suggests that SST PC inhibits TNF- α -induced NF- κ B activation in a PI3K- and JAK-2-dependent pathway.

3.3. SST PC modulates I κ c expression induced by TNF- α

Western blotting revealed that TNF- α induced I κ c expression by HUVECs (Lanes 1 and 2, Fig. 3). NAC had no effects on TNF- α -induced I κ c expression (data not shown). However, SST PC had bimodal effects on TNF- α -induced I κ c expression. SST PC had significant inhibitory effects on TNF- α -induced I κ c expression at 10^{-8} M (S1, Fig. 3), but the effects were not significant at 10^{-10} M (S2, Fig. 3). AG-490 (A + S, Fig. 3) and wortmannin (W + S, Fig. 3) reversed the inhibitory effects of SST PC on TNF- α -induced I κ c expression, whereas PD98059 had no significant effects (P + S, Fig. 3).

4. Discussion

This study demonstrated that SST PC can attenuate TNF- α -induced release of IL-6 and IL-8, which are indicative of endothelial damage. NF- κ B and I κ c signaling are possibly the pathways responsible. These observations confirm that SST PC exists in human endothelial cells and may contribute, at least in part, to *in vivo* infarct reduction in animal models of SST PC.³

Activation of G-protein-linked phospholipase C, tyrosine kinase pathways and protein kinase C and the generation of oxygen radicals are known to be involved in PC.¹⁵ ATP-sensitive potassium channels are widely considered to be the most probable triggers and effectors of PC.¹⁵ Recent studies suggest that mitoK_{ATP} in addition to sarcolemmal K_{ATP} channels, might mainly account for the protective mechanism.^{15–18} It has been proved that mitoK_{ATP} channel opening is related to mitochondrial swelling and optimization of respiration,¹⁶ prevention of mitochondrial calcium overload,¹⁷ and control of reactive oxygen radicals,¹⁸ all of which are possible links to cardioprotection during severe myocardial ischemia and infarction.

As mentioned above, the cellular targets of PC seem to be heterogeneous. Although saline-perfused isolated hearts or isolated cardiomyocytes can be preconditioned,^{19,20} a so-called microvascular component of PC has been proposed.^{21–23} This concept involves reduction of post-ischemic inflammation or the mitigation of leukocyte adhesion in the perfused vascular bed after PC. Endothelial cells, the interface between parenchymal tissue and blood cells, are involved in this significant pathophysiological process.

We first demonstrated that SST analogs may mimic ischemic preconditioning in a rat model of myocardial infarction.³ However, it is not known whether this cardioprotective effect can be attributed to either myocardial PC or endothelial PC. SST is produced by neuroendocrine, inflammatory, and immune cells in response to various mediators.²⁴ Its receptors are a family of seven-transmembrane (TM)-domain G-protein-coupled receptors that comprise five distinct subtypes termed SST receptor (SSTR) 1–5 encoded by separate genes on different chromosomes.^{24–27} It would be interesting to investigate if SST analogs can exert protective effects in endothelium via their specific receptors. The data from this study show that an SST analog can protect HUVECs from TNF- α -induced damage.

It has been reported that the physiological effects of SST and SSTRs depend on insulin-like growth factor-1 (IGF-I) in many tissues.^{28,29} It has been reported that IGF-I leads to coronary arteriolar dilation through activation of potassium channels,³⁰ induction of nitrous oxide,³¹ and reduction of intracellular calcium from vascular endothelium.³² IGF-I is also known to increase myocardial contraction,³¹ but excessive IGF-I may also induce cardiac hypertrophy and impaired cardiac function, as in individuals with acromegaly.³³ Although our study did not explore the role of IGF-I in endothelial SST PC, we may consider elucidation of the link between NF- κ B/I κ c activation and IGF-I formation.

As SST is produced by many tissues and has various physiological effects, its five SSTRs share common signaling pathways, such as inhibition of adenylyl cyclase, activation of phosphotyrosine phosphatase, and modulation of MAPK through G-protein-dependent mechanisms. Some of the subtypes are also coupled to inward rectifying K⁺ channels, to voltage-dependent Ca²⁺ channels, a Na⁺/H⁺ exchanger, AMPA/kainate glutamate channels, phospholipase C, and phospholipase

A2.²⁴ Our previous study showed that acute effects of SST PC involve novel signaling mechanisms, including activation of protein kinase C, tyrosine kinases, and mitoK_{ATP} channels, instead of systemic IGF-I activation.³ This differs from the mechanisms mentioned above. So far, many possible physiological mechanisms for SST analogs have been identified.^{24,34,35} These may involve various actions of different SSTR subtypes and the diverse distribution of these SSTRs in different tissues. However, AP-1³⁶ or NF- κ B,³⁷ as transcription factors, may play a central role in regulation. Todisco et al³⁶ demonstrated that SST inhibits AP-1 function via multiple protein phosphatases and identified the mechanisms by which SST inhibits immediate early gene expression. Anhod et al³⁷ showed that SST affects TNF- α -induced IL-6 and IL-8 secretion from pancreatic myofibroblasts and that SST inhibits TNF- α -induced IL-6 secretion in a dose-dependent manner. Although these reports were for different tissues or cell lines, the signaling mechanism may be similar, especially since the five SSTRs share common pathways. The two studies demonstrate that AP-1 and NF- κ B could be candidate factors. It is reasonable to suppose that these two critical components could be responsible for SST-related endothelial PC, especially since our results implicate NF- κ B. Our study demonstrates that TNF- α increases endothelial release of IL-6 and IL-8 in NF- κ B/I κ k-dependent pathway. SST PC attenuated TNF- α -induced IL-6 and IL-8 release and was involved in attenuation of TNF- α -related NF- κ B activation. An inhibitor study demonstrated that PI3K and possibly JAK-2 may be involved in this cellular signaling. PI3K and possibly JAK-2 may be the major signaling pathways involved in modulatory effects of SST on TNF- α -related I κ k activation.

In conclusion, SST PC modulates I κ k in a PI3K- and JAK-2-dependent pathway, which in turn attenuates activation of NF- κ B induced by TNF- α in HUVECs.

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